UNITED STATES DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESEARCH SERVICE
VETERINARY BIOLOGICS DIVISION
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SAM 107

Standard Requirements	Date	Agents
V-32, V-56, V-59	June 14, 1971	Bovine Rhinotracheitis Bovine Virus Diarrhea, and Parainfluenza-3

SUPPLEMENTAL ASSAY METHOD

FOR

TITRATION OF NEUTRALIZING ANTIBODY

(Constant Serum - Varying Virus Method)

A. SUMMARY

This is an *in vitro* assay method which employs a cell culture system for determining the antibody titer of serum against Bovine Rhinotracheitis (IBR), Bovine Virus Diarrhea (BVD), and Parainfluenza-3 (PI-3) viruses.

B. MATERIALS

1. Cell Cultures Roller tubes (16 x 150 mm) containing monolayers of primary bovine embryonic kidney (BEK) cells are used for IBR and PI-3 serum neutralization (SN) tests, and Leighton tubes containing monolayer BEK cells on coverslips (10.5 x 35 mm) are used for BVD SN tests, Cells found free from extraneous agents are used in these tests.

a. Primary BEK cells are grown from trypsinized kidney cortical tissue, frozen, and stored at -80 C, and tested for extraneous agents.

b. Frozen cells are thawed, suspended in growth medium (Appendix, No. 1), and 1 ml amounts dispensed into

Leighton or roller tubes.

c. The tubes containing the cells are incubated in stationary racks at 36 to 37 C until the monolayer is at least 80% confluent. The growth medium is

replaced with maintenance medium (Appendix, No. 2)

just before the tubes are inoculated.

2. Indicator Viruses Veterinary Biologics Division reference

IBR, BVD, or PI-3 viruses are used.

3. Diluent Maintenance medium, without serum, is used to

make dilutions of the virus and serum.

4. Test Serums

a. Serums to be tested.

b. Negative control serum.

5. Conjugate Veterinary Biologics Division conjugated BVD specific immune serum is used in the BVD SN test system.

6. Guinea Pig Red Blood Cells (RBC) for the Hemadsorption (Had) Test

a. Blood from healthy guinea pigs is collected aseptically in an equal volume of sterile Alsever's

solution (Appendix, No. 3).

b. The RBC are washed 3 times in Alsever's solution and sedimented each time by centrifugation at 1,000 rpm (250 G's) for 15 minutes.

c. The RBC are stored at 5 C as a 50% suspension in Alsever's solution.

d. For the hemadsorption test, the RBC are diluted to a 0.5% suspension in phosphate buffered saline (PBS) (Appendix, No. 4)

C. METHOD

1. Dilution of Indicator Virus Serial tenfold dilutions of the indicator virus are made in sterile tubes (16 x 150 mm) containing diluent. The number of dilutions depends upon the predetermined titer of the indicator virus. A separate pipette is used to make each virus dilution, and care taken to not touch the diluent with the end of the pipette. Each virus dilution is mixed with a Vortex* or similar type mixer. The tenfold dilutions are made as follows:

a. Nine ml diluent is placed in a series of tubes,

starting with 10-1.

b. One ml virus is added to the 10-1 tube. Pipette is discarded and the contents mixed.

c. One ml of the 10-1 dilution is added to the 10-2 tube. Pipette is discarded and the contents mixed.

d. This process is continued until the desired number of virus dilutions are made.

2. Serum Neutralization of Virus All serums are heat-inactivated at 56 C for 30 minutes.

a. For each test serum, a series of tubes is placed in parallel to the virus dilution tubes. Another series is included for a normal serum.

b. Starting with the highest dilution, 1 ml of each virus dilution is placed into its corresponding tube in each series of tubes.

*No endorsement expressed or implied

c. One ml of undiluted serum is added to all tubes in a series containing the virus dilutions, mixed, and incubated at room temperature for 45 minutes. Care is taken to avoid foaming when mixing.

d. Five cell culture tubes are inoculated with 0.2 ml of each serum-virus dilution. One pipette may be used for each series of tubes by starting with the highest dilution and progressing through the lowest.

e. Five uninoculated cell culture tubes are incubated and processed along

with the other cultures as a check on the test system.

3. Interpretation The 50% endpoint of each serum is calculated by the method of

Reed and Muench or Spearman-Karher, The neutralization index (NI) is then

determined by subtracting the log of the titer obtained with the immune serum

from the titer obtained with the normal serum.

Example: Log TCID50 titer with normal serum 6.0

Log TCID50 titer with immune serum - -2.7

NI = 3.3

The cells in the uninoculated control tubes must remain normal.

4. Incubation and Reading of Tests for Three Viral Agents

a. Bovine Rhinotracheitis

The inoculated BEK roller tubes are incubated at 35 to 37 C for 4 to 6 days. The tubes are examined for cytopathic effect (CPE) typical of IBR virus. The number of tubes found positive and negative for CPE are recorded and the 50% endpoints calculated. Then, the neutralization index is determined.

b. Bovine Virus Diarrhea

The inoculated Leighton tubes are incubated at 35 to 37 C for 4 to

6 days. The coverslips are removed from the tubes and processed for reading by the fluorescent antibody (FA) technique. The cells on the coverslips are stained as follows:

(1) Coverslips are removed from the tubes and placed in racks.

(2) They are rinsed in PBS, then in distilled water, and dried.

(3) They are fixed in cold acetone for 15 minutes, then dried thoroughly.

(4) The cells are covered with conjugated BVD specific immune serum and held in a high humidity incubator at 37 C for 30 minutes.

(5) Conjugate is drained and the coverslips are washed in a gently circulating PBS bath for 10 minutes, rinsed in distilled water, and dried.

(6) Coverslips are mounted on glass slides with the cells down using FA mounting fluid.

Monolayer cells are examined by fluorescence microscopy with dry darkfield condenser. The number of slides positive and negative for fluorescence are recorded and the 50% endpoints calculated. Then, the neutralization index is determined.

c. Parainfluenza-3

The inoculated BEK roller tubes are incubated at 35 to 37 C for 4 to 6 days. The cell layers are examined by one or both of the following methods:

(1) Cytopathic effect:

The tubes are examined for CPE typical of PI-3 virus. The number of tubes found positive and negative for CPE are recorded and the 50% endpoints calculated.

(2) Hemadsorption test:

(a) Fluids are poured from the tubes.

(b) The cells are washed once with PBS.

(c) To each tube is added 1 ml of a 0.5% suspension of RBC.

(d) The tubes are placed so that the cell monolayer is covered with the RBC suspension and allowed to stand 15 to 20 minutes at room temperature.

(e) The suspension of RBC is poured off and the monolayers are washed 3 times with PBS.

(f) The PBS is drained from the tubes and the monolayers are examined microscopically for hemadsorption.

The number of tubes positive and negative for HAd are recorded and the 50% endpoints calculated. Then, the neutralization index is determined.

APPENDIX

1. Growth Medium

Lactalbumin hydrolysate		0.5%
Hanks BSS q.s. ad		100.0%
Antibiotics - Penicillin		100 units/ml
	Streptomycin	100 mcg/ml
	Kanamycin	160 mcg/ml
	Amphotericin B	2 mcg/ml

Ten percent fetal calf serum is added.**

2. Maintenance Medium

Lactalbumin hydrolysate		0.5%
MEM (Eagle)* q.s. ad		100.0%
Antibiotics - Penicillin		100 units/ml
	Streptomycin	100 mcg/ml
	Kanamycin	160 mcg/ml
	Amphotericin B	2 mcg/ml

Two percent fetal calf serum is added.**

3. Alsever's Solution

Dextrose	2.05%
Sodium citrate	0.8%
Sodium chloride	0.42%
Citric acid	0.55%
Distilled H20 q.s. ad	100.0%

*Available from GIBCO, Catalog No. F-15. No endorsement expressed or implied.

**Serum previously tested and found negative for extraneous agents.

4. Phosphate Buffered Saline (PBS-Dulbecco)

NaC1	0.9%
KC1	0.02%
Na2HP04	0.115%
KH2PO4	0.02%
CaCl2 (anhy.)	0.01%
MgCl2.6H20	0.01%
Distilled H20 q.s. ad	100.0%

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